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**New peptide boronic acid derivs. inhibiting serine proteins - e.g.
thrombin, used for trypsin-like serine protease and treating related
disorders**

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(METT-I); NOVARTIS AG (NOVS); SANDOZ ERFindungen VERW GMBH (SANO);
SANDOZ PATENT GMBH (SANO); SANDOZ SA (SANO)

Inventor: METTERNICH R; METTERNISCH R

Number of Countries: 029 Number of Patents: 021

Patent Family:

Patent No	Kind	Date	Applicat No	Kind	Date	Week	
EP 471651	A	19920219	EP 91810626	A	19910809	199208	B
AU 9181792	A	19920220				199218	
CA 2048953	A	19920214				199218	
FI 9103788	A	19920214	FI 913788	A	19910809	199219	
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JP 4330094	A	19921118	JP 91201750	A	19910812	199301	
ZA 9106393	A	19930428	ZA 916393	A	19910813	199323	
NO 9200257	A	19930721	NO 92257	A	19920120	199338	N
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AU 643312	B	19931111	AU 9181792	A	19910812	199401	
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NO 300504	B1	19970609	NO 92257	A	19920120	199730	N
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Priority Applications (No Type Date): GB 9017694 A 19900813; NO 92257 A
19920120; RO 200147 A 19920213

Patent Details:

Patent No	Kind	Lan	Pg	Main IPC	Filing Notes
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Designated States (Regional): AT BE CH DE ES FR GB GR IT LI LU NL SE

JP 4330094	A	16	C07K-005/06	
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ZA 9106393	A	41	C07C-000/00	
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AU 643312	B		C07F-005/02	Previous Publ. patent AU 9181792
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US 5288707	A	12	A61K-037/02	Cont of application US 91743847
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JP 2539965	B2	19	C07K-005/062	Previous Publ. patent JP 4330094
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NO 300504	B1		C07K-005/06	Previous Publ. patent NO 9200257
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RU 2075481	C1	14	C07K-005/06
FI 9103788	A		C07K-005/06
HU 59161	T		C07K-005/04
PT 98638	A		C07K-007/00
CS 9102484	A2		C07K-007/00
NO 9200257	A		C07K-005/06
NZ 239359	A		C07K-005/04
RO 107661	B1		C07K-007/06
IL 99163	A		C07K-005/06
TW 275634	A		C07K-005/00
PH 29066	A		A61K-037/02

Abstract (Basic): EP 471651 A

Peptide boronic acids of formula (I) are new. W = H or an N-protecting gp., Y = a sequence of n amino acids (AA) such that the n+1 AA peptide Y-Lys or Y-Arg has an affinity for the active site of a trypsin-like protease and at least one of the AA is unnatural (UAA) and has a hydrophobic side chain, n = 1-10, Q1, Q2 = OH, COR1, CONR1R2, NR1R2 or OR3 or Q1, Q2 together = a diol residue, R1-R3 = 1-10C alkyl, 6-10C aryl, 6-10C aralkyl, or phenyl (substd. by 1-3 of 1-4C alkyl, halogen or 1-4C alkoxy), R4 = H or 1-10C alkyl, R5 = AX, 6-10C aryl or 6-10C aralkyl or R4, R5 = (CH2)3, A = (CH2)z, CHMe(CH2)2, CH2CHMeCH2, (CH2)2CHMe, (CH2)2CMe2, CHMe(CH2)3, CH2CHMe(CH2)2, (CH2)2CHMeCH2, (CH2)3CHMe or (CH2)3CMe2, z = 2-5, X = NH2, NHC(NH)NH2, SC(NH)NH2, N3, 1-4C alkoxy, 1-4C alkylthio and asterisk = an asymmetric C atom, D-, L- or a mixt.

Also claimed are UAA of formula NH2CHR11COOH (II), R11 = CH2SiMe3, CH2CMe3, p-hydroxybenzyl or its t-BuPh2Si ether, adamantyl or 1-adamantylmethyl.

USE - (I) are inhibitors of trypsin-like serine proteases e.g. thrombin, factor Xa, kallikrein, plasmin, prolyl endopeptidase and IgA1 protease, with superior properties e.g. oral activity, rapid onset, and low toxicity. The thrombin or Xa inhibitors have antithrombogenic properties and may be used for internal admin. for extracorporeal blood loops or for collection or distribution containers, tubing or implantable appts. coming into contact with blood, to prevent coagulation. (19pp Dwg.No.0/0)

Abstract (Equivalent): US 5288707 A

Borolysine peptidomimetics of formula WY-N(R)-CHR'-BQQ' (I) are new. In (I), W is H or N-protective gp., Y is an aminoacid sequence with 1-10 units contg. at least one hydrophobic side chain, such that the sequence Y-Lys or Y-Arg has an affinity for the active site of a trypsin-like protease, Q and Q' are identical or different, denoting OH, OQ'', COQ'', CON(Q'')2 or N(Q'')2, where each Q'' is 1-10C alkyl, 6-10C aryl or aralkyl, or opt. substd. Ph, or Q and Q' together denote -O-Z-O-, where Z is the rest of a linear or alicyclic gp., R is H or 1-10C alkyl, and R' is -AX, where A is a linear or branched 2-5C alkylene and X is NH2, NH-C(=NH)-NH2, S-C(=NH)-NH2, N3, 1-4C alkoxy or alkylthio or SiMe3, or R and R' together denote (CH2)3, forming a closed ring, and the asymmetric C has a D- or L-configuration.

USE - Cpd. (I) are potent thrombin inhibitors for the treatment of hypertensive thrombotic conditions, malignant tumours, inflammatory diseases etc.

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Intellectual Property Office of New Zealand
IP Summary Report

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New Zealand Patent 335543 Page 1 of 36 printed on Saturday 7th May 2005

(61) Classification: A61K38/43		Status: 70 Accepted	Version number: 5 IP type: Patent Convention
		Client Ref: JP801966	
(22) NZ Filing date: 03 May 1999 (30) Priority Data: (31) 96 619280 (32) 18 March 1996 (33) US (71) Applicants: LUDWIG INSTITUTE FOR CANCER RESEARCH, 605 Third Avenue, New York, New York 10158, United States of America BOEHRINGER INGELHEIM INTERNATIONAL GmbH, D-6507 Ingelheim am Rhein, Federal Republic of Germany (72) Inventors: Zimmerman, Rainer Park, John E Old, Lloyd J Rettig, Wolfgang Contact: BALDWIN SHELSTON WATERS, Level 14, NCR House, 342 Lambton Quay, Wellington, New Zealand Primary Examiner: CLAIRE MCINNES Journal: 1461		(62) Divided out of: 331758 Divisional number(s): 509792 Date actions completed: Application Accepted: 15 March 2001 Next renewal date: 03 May 2003	
Office title: Use of a collagen derivative as an fibroblast activation protein alpha (FAP-alpha) inhibitor (54) Applicant title: Isolated dimeric fibroblast activation protein alpha, and uses thereof (57) Abstract: Patent 335543 Use of an inhibitor of FAP-alpha activity which interacts with molecules having FAP-alpha activity, said inhibitor being a collagen derivative, in the manufacture of a medicament for treating a subject with a pathological condition characterized by abnormal FAP-alpha activity.			

**** End of report ****

335543

Patents Form No. 5

Our Ref: JP801966

NEW ZEALAND
PATENTS ACT 1953

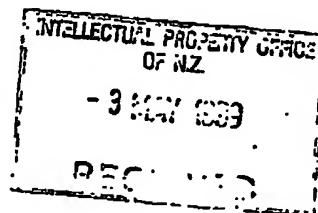
COMPLETE SPECIFICATION

**DIVISIONAL APPLICATION OUT OF
NEW ZEALAND PATENT APPLICATION NO. 331768
FILED ON 12 MARCH 1997**

We, BOEHRINGER INGELHEIM INTERNATIONAL GMBH a body corporate organised under the laws of Germany of Corporate Patent Department, D-6507, Ingelheim Am Rhein, Fed Republic Of Germany, Fed Republic Of Germany and **LUDWIG INSTITUTE FOR CANCER RESEARCH**, a research institute of 605 Third Avenue, New York, New York 10158, United States of America

hereby declare the invention, for which We pray that a patent may be granted to us and the method by which it is to be performed, to be particularly described in and by the following statement:

PT0540153



335543

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PCT/US97/04215

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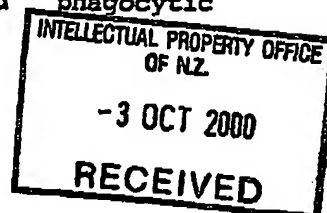
ISOLATED DIMERIC FIBROBLAST ACTIVATION
PROTEIN ALPHA, AND USES THEREOF

FIELD OF THE INVENTION

This invention relates to certain molecules associated with cancer tissues and reactive tumor stromal cells. More particularly, it relates to fibroblast activation protein alpha ("FAP α " hereafter) molecules. A monomeric form of the molecule has previously been identified immunochemically, but nucleic acid molecules coding for it had not been isolated or cloned nor have dimers been identified. These, *inter alia*, are features of the invention. The monomeric protein has a molecular weight of from about 88 to about 95 kilodaltons as determined by SDS-PAGE of boiled samples. The dimer has a molecular weight of about 170 kilodaltons as determined by SDS-PAGE of unboiled samples. FAP α is characterized by a number of features and properties which are shared by and characteristic of membrane bound enzymes, suggesting very strongly that it, too, is a membrane bound enzyme. The nucleic acid molecules, which are a key part of the invention, are useful both as probes for cells expressing FAP α , and as starting materials for recombinant production of the protein. The FAP α protein can then be used to produce monoclonal antibodies specific for the protein and are thus useful diagnostic agents themselves. They also have additional uses, including uses related to enzymatic functions, as described herein.

BACKGROUND AND PRIOR ART

The invasive growth of epithelial cancers is associated with characteristic cellular and molecular changes in the supporting stroma. For example, epithelial cancers induce the formation of tumor blood vessels, the recruitment of reactive tumor stromal fibroblasts, lymphoid and phagocytic



infiltrates, the release of peptide mediators and proteolytic enzymes, and the production of an altered extracellular matrix (ECM). See, e.g., Folkman, Adv. Cancer Res. 43: 175-203 (1985); Basset et al., Nature 348: 699-704 (1990); Denekamp et al., Cancer Metastasis Rev. 9: 267-282 (1990); Cullen et al., Cancer Res. 51: 4978-4985 (1991); Dvorak et al., Cancer Cells 3: 77-85 (1991); Liotta et al., Cancer Res. 51: 5054s-5059s (1991); Garin-Chesa et al., J. Histochem. Cytochem. 37: 1767-1776 (1989). A highly consistent molecular trait of the stroma in several common histologic types of epithelial cancers is induction of the fibroblast activation protein (FAP α), a cell surface glycoprotein with an observed M_r of 95,000 originally discovered with a monoclonal antibody, mAb F19, raised against proliferating cultured fibroblasts. See Rettig et al., Cancer Res. 46: 6406-6412 (1986); Rettig et al., Proc. Natl. Acad. Sci. USA 85: 3110-3114 (1988); Garin-Chesa et al., Proc. Natl. Acad. USA 87: 7235-7239 (1990); Rettig et al., Cancer Res. 53: 3327-3335 (1993). Each of these four papers is incorporated by reference in its entirety.

Immunohistochemical studies such as those cited supra have shown that FAP α is transiently expressed in certain normal fetal mesenchymal tissues but that normal adult tissues are generally FAP α ⁻. Similarly, malignant epithelial, neural and hematopoietic cells are generally FAP α ⁻. However, most of the common types of epithelial cancers, including >90% of breast, lung, skin, pancreas, and colorectal carcinomas, contain abundant FAP α ⁺ reactive stromal fibroblasts. Garin-Chesa et al., Proc. Natl. Acad. Sci. USA 87: 7235-7239 (1990). The FAP α ⁺ tumor stromal fibroblasts almost invariably accompany tumor blood vessels, forming a distinct cellular compartment interposed between the tumor capillary endothelium and the basal aspect of malignant epithelial cell clusters. While FAP α ⁺ stromal fibroblasts are found in both primary and metastatic carcinomas, benign and premalignant epithelial lesions, such as fibroadenomas of the breast and colorectal adenomas only rarely contain FAP α ⁺ stromal cells. In contrast

to the stroma-specific localization of FAP α in epithelial neoplasms, FAP α is expressed in the malignant cells of a large proportion of bone and soft tissue sarcomas. (Rettig et al., Proc. Natl. Acad. Sci. USA 85: 3110-3114 (1988)). Finally, FAP α ⁺ fibroblasts have been detected in the granulation tissue of healing wounds (Garin-Chesa et al., *supra*). Based on the restricted distribution pattern of FAP α in normal tissues and its uniform expression in the supporting stroma of many epithelial cancers, clinical trials with ¹²⁵I-labeled mAb F19 have been initiated in patients with metastatic colon cancer (Welt et al., Proc. Am. Assoc. Cancer Res. 33: 319 (1992); Welt et al. J. Clin. Oncol. 12: 1561-1571 (1994)) to explore the concept of "tumor stromal targeting" for immunodetection and immunotherapy of epithelial cancers.

Rettig et al., Int. J. Cancer 58: 385-392 (1994), incorporated by reference, discusses the FAP α molecule and its features. Rettig et al postulate that FAP α is found in high molecular weight complexes in excess of 400 kilodaltons, but do not discuss the possibility of dimeric molecules, nor does the paper elaborate on the specific enzymatic properties of the molecule.

The induction of FAP α ⁺ fibroblasts at times and sites of tissue remodeling during fetal development, tissue repair, and carcinogenesis is consistent with a fundamental role for this molecule in normal fibroblast physiology. Thus, it is of interest and value to isolate and to clone nucleic acid molecules which code for this molecule. This is one aspect of the invention, which is described in detail together with other features of the invention, in the disclosure which follows. Further aspects of the invention include the dimeric FAP α molecules, and the exploitation of the properties of these molecules. These features are also elaborated upon hereafter.

The applicant's related parent application NZ 331758 provides an isolated, dimeric FAP α molecule, having a molecular weight of about 170 kilodaltons as determined by SDS-PAGE, wherein said dimeric FAP α molecule is capable of degrading extracellular matrix proteins.

(Followed by page 3a)

3a

NZ 331758 also provides a method for cleaving a terminal dipeptide of formula Xaa-Pro from a molecule, comprising contacting said molecule with a second molecule, said second molecule having FAP α enzymatic activity.

NZ 331758 also provides a method for identifying a substance which interacts with a molecule having FAP α activity, comprising combining said molecule with a sample to be tested, and determining any interaction with said molecule as an indication of a molecule which interacts with a molecule having FAP α activity.

NZ 331758 also provides a method for determining if a substance interacts with a molecule having FAP α activity, comprising combining said substance and said molecule with Ala-Pro-AFC, determining interaction of said molecule with Ala-Pro-AFC, and comparing said interaction to interaction of said molecule with Ala-Pro-AFC in the absence of said substance, wherein a difference therebetween indicates that said substance interacts with said molecule.

NZ 331758 also provides fusion protein comprising a portion of an FAP α molecule sufficient to retain FAP α activity and a non FAP α amino acid sequence, wherein said fusion protein is water soluble.

The present, divisional application provides a method for treating a subject with a pathological condition characterized by abnormal FAP α activity, comprising administering to a subject in need thereof an amount of a substance which interacts with molecules having FAP α activity sufficient to normalize the FAP α activity level in said subject.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 compares the deduced amino acid sequence for FAP α , and the known sequence of CD26. The alignment has been optimized.

(followed by page 4)

Figures 2A-2H, inclusive, display immunohistochemical detection of FAP α and CD26 in various tissues. In figures 2A and 2B, breast cancer is studied, for FAP α (figure 2A), and CD26 (figure 2B). In figures 2C and 2D, malignant fibrous histiocytoma is studied, for FAP α (figure 2C), and CD26 (figure 2D). Dermal scar tissue is examined in figures 2E (FAP α), and 2F (CD26). Renal cell carcinoma is studied in figure 2G (FAP α), and 2H (CD26).

Figure 3 presents some of the data generated in experiments which showed that FAP α had extracellular matrix (ECM) protein degrading activity. When zymographic detection of gelatin degrading extracts of 293-FAP was carried out, the active substance was found to have a molecular weight of about 170 kD, via SDS-PAGE, using unboiled samples to preserve enzyme activity.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

Fibroblast cell line WI-38 had been observed, previously, to react with mAb F19 (Rettig et al., Canc. Res. 46: 6406-6412 (1986); Rettig et al., Proc. Natl. Acad. USA 85: 3110-3114 (1988); Garin-Chesa et al., Proc. Natl. Acad. Sci. USA 87: 7235-7239 (1990); Rettig et al., Canc. Res. 53: 3327-3335 (1993)). It was used in the experiments which follow.

A cDNA library was prepared from WI-38, using well known techniques and commercially available materials. Specifically, the library was constructed in expression vector pCDNA1, using the Fast Track mRNA isolation kit, and Librarian cDNA phagemid system. Once the library was prepared, the vectors were electroporated into cell line *E. coli* MC 1061/P3. The pCDNA1 expression vector contains an antibiotic resistance gene, so the *E. coli* were selected via antibiotic resistance. The colonies which were resistant were then used in further experiments. The plasmid DNA from the colonies was obtained via alkaline lysis and purification on CsCl₂, in accordance with Sambrook et al, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Lab, Cold Spring Harbor, N.Y. 2d Ed. 1989). The technique is well known to the art, but is

incorporated by reference herein.

Once the plasmid DNA was isolated, it was used to transfect COS-1 cells, which were then cultured for forty-eight hours, after which these were tested with antibody coated dishes. The mAbs used included F19, as described by Rettig et al., (1986), supra, which is incorporated by reference in its entirety. As COS-1 cells are normally FAP α , any positive results indicated the presence of the coding sequence. The immunoselection protocol was that of Aruffo et al., Proc. Natl. Acad. Sci USA 84: 3365-3369 (1987), incorporated by reference herein.

Plasmid DNA from positive clones was recovered, in accordance with Hirt, J. Mol. Biol. 26: 365-369 (1967), reintroduced into *E. coli* MC 1061/P3, and reselected in COS-1 cells.

The protocol presented herein was followed for four rounds. After this, the plasmid DNA of 50 isolated bacterial colonies was purified, using the Qiagen plasmid kit. Of the colonies, 27 clones were found to contain identical 2.8 kb inserts, as determined by EcoRI restriction enzyme mapping. Several of these were found to contain FAP α -specific cDNA as determined by transient expression in COS-1 cells and direct immunofluorescence staining with mAb F19. One of these clones, i.e., "pFAP.38" was selected for further study, as elaborated upon infra.

Example 2

Once pFAP.38 had been identified, it was tested together with a vector coding for known cell surface marker CD26 ("pCD26"), as well as with control vector pCDNA I.

In these experiments, COS-1 cells were transfected with one of pFAP.38, pCD26, or pCDNAI. After forty-eight hours, the transfectants were tested, using the well known MHA rosetting assay for cell surface antigen expression. In these experiments, mAb F19, which is FAP α specific, was used, together with mAb EF-1, which is CD26 specific. Also used were four other FAP α specific mAbs, i.e., FB23, FB52, FB58 and C48. Also tested were two cancer cell lines, which are known

to react with mAb F19 (SW872 liposarcoma), or EF-1 (SK-OV6 ovarian cancer). The results are set forth in Table 1, which follows.

Table 1. Cell surface expression of multiple FAP α epitopes and CD26 in human cells and COS-1 cell transfectants

Target cell	Cell surface antigen expression					
	F19	FB23	FB52	FB58	C48	EF-1
<u>Human cells</u>						
SW872 liposarcoma	>95%	>95%	>95%	>95%	>95%	-
SK-OV6 ovarian cancer	-	-	-	-	-	>95%
<u>COS-1 transfectants</u>						
COS-pCDNAI control	-	-	-	-	-	-
COS-pFA P 38	40%	30%	40%	20%	20%	-
COS-pCD26	-	-	-	-	-	40%

Example 1

Immunoprecipitation studies were then carried out to identify the antigen being targeted by the antibodies.

Cells were metabolically labelled with Trans ^{35}S -label, (ICN), extracted with lysis buffer (0.01 M Tris-HCl/0.15 M NaCl/0.01 M MgCl₂/0.5% Nonidet P-40/aprotinin (20 ug/ml)/2 mM phenylmethyl- sulfonyl fluoride), and then immunoprecipitated. The protocols used are all well known, as will be seen by reference to Rettig et al., Canc. Res. 53: 3327-3335 (1993); and Fellingner et al., Canc. Res. 51: 336-340 (1991), the disclosures of which are all incorporated by reference in

their entirety. Precipitating mAbs were negative control mouse Ig, mAb F19, or EF-1. Control tests were carried out with mock transfected COS-1 cells. Following immunoprecipitation, the immunoprecipitates were boiled in extraction buffer and separated by NaDodSO₄/PAGE, under reducing conditions. In some experiments, an additional test was carried out to determine whether or not the immunoprecipitated material was glycosylated. In these experiments, cell extracts were fractionated with Con A-SEPHAROSE prior to immunoprecipitation. Following immunoprecipitation, but prior to fractionation on NaDodSO₄/PAGE, these precipitates were digested with N-Glycanase.

The results showed that, in COS-1 cells, pFAP.38 directs expression of an 88 kd protein species (as determined via SDS-PAGE), which is slightly smaller than the 95 kd FAP α species produced by SW872, or cultured fibroblasts. Digestion with N-Glycanase produced peptides of comparable size (i.e., 74 kd versus 75 kd), showing that the glycosylation of the FAP α protein in COS-1 cells is different than in the human cell lines.

Example 4

Classic Northern blot analysis was then carried out, using the mRNA from FAP α fibroblast cell lines WI-38 and GM 05389, and FAP α ovarian cancer cell line SK-OV6. Using the procedures of Sambrook et al., *supra*, five micrograms of mRNA from each cell line were tested. The probes used were ³²P labelled, and were prepared from a 2.3 kb ECO I fragment of pFAP.38, a 2.4 kb Hind III fragment of CD26, and a 1.8 kb BamHI fragment of γ -actin cDNA. These fragments had been purified from 1% agarose gels.

The extracts of FAP α fibroblast strains showed a 2.8 kb FAP mRNA species, but extracts of SK-OV6 do not. A γ -actin mRNA species (1.8 kb), was observed in all species.

Example 5

The cDNA identified as coding for FAP α was subjected to more detailed analysis, starting with sequencing. The classic

Sanger methodology, as set forth in Proc. Natl. Acad. Sci. USA 74: 5463-5467 (1977), was used to sequence both strands of the cDNA. Once this was secured, an amino acid sequence was deduced therefrom. This information is presented in SEQ ID NO: 1. The sequence was then compared to the known amino acid sequence of CD26 (Morimoto et al., J. Immunol. 143: 3430-3437 (1989)). Figure 1 presents the comparison, using optimized sequence alignment. Any gaps in the comparison are indicated by asterisks, while identical amino acids are shown by dashes in the CD26 sequence. A hydrophobic, putative transmembrane sequence is double underlined, while potential N-glycosylation sites are single underlined.

The sequence analysis shows a 2812 base pair insert, wherein 2277 base pairs constitute the open reading frame. This ORF extends from start codon ATG at nucleotide 209, to stop codon TAA at 2486.

The deduced polypeptide is 760 amino acids long, and has a molecular weight of 87,832. In contrast, N-Glycanase digested, immunopurified FAP α was reported to have an estimated M_r of 75,000 on NaDodSO₄/PAGE (Rettig et al., Canc. Res. 53: 3327-3335 (1993)). A GenBank data base search was carried out. The most closely related genes found were those encoding dipeptidyl peptidase IV homologues (DPPIV; EC 3.4.14.5), with human DPPIV (also known as T-cell activation antigen CD26), showing 61% nucleotide sequence identity, and 48% amino acid sequence identity.

The second set of related genes are human, rat, and bovine homologues of DPPX, a gene of unknown function widely expressed in brain and other normal tissues. The predicted human DPPX gene product shows about 30% amino acid sequence identity with FAP α and CD26. The FAP α molecule exhibits structural features typical of type II integral membrane proteins, including a large COOH-terminal extracellular domain, a hydrophobic transmembrane segment, and a short cytoplasmic tail. The putative extracellular domain contains five potential N-glycosylation sites, eleven cysteine residues (eight of which are conserved between FAP α and CD26), and

three segments corresponding to highly conserved catalytic domains characteristic of serine proteases, such as DPPIV. These conserved sequences are presented in Table 2, which follows. Comparisons to DPPIV and DPPX were made via Morimoto et al., supra; Wada et al., Proc. Natl. Acad. Sci. USA 89: 197-201 (1992); Yokotani et al., Human Mol. Genet. 2: 1037-1039 (1993).

Example 6

An additional set of experiments were carried out to determine whether FAP α related sequences are present in non-human species. To do so, human, mouse, and Chinese hamster genomic DNA was digested using restriction enzymes, and tested, via Southern blotting, using the 2.3 kb fragment, labelled with ^{32}P , describes supra. Hybridization was carried out using stringent washing conditions (0.1 x SSC, 0.1% NaDodSO $_4$, 68°C). Cross-hybridization was readily observed with both the mouse and hamster DNA, suggesting the existence of highly conserved FAP α homologues. In control experiments using the CD26 cDNA fragment described supra, no evidence of cross hybridization was observed.

Example 7

The CD26 molecule shares a number of biochemical and serological properties with FAPB, which is a previously described, FAP α associated molecule having a molecular weight of 105 kd, and is found on cultured fibroblasts and melanocytes (Rettig et al., Canc. Res. 53: 3327-3335 (1993)). Cotransfection experiments were carried out to determine whether FAPB is a CD26 gene product. To test this, the same protocols were used which were used for transfection with pFAP.38 or pCD26, as described supra, but using the two vectors. The results presented supra showed that cotransfection efficiency was about 40% for each vector, so about 10-20% of cell should be cotransfected.

Following cotransfection, the COS-1 cells were Trans ^{35}S -labeled, as described supra, then lysed, also as described supra.

The resulting cell extracts were separated on Con A

SEPHAROSE, and the antigen (FAP α and/or CD26) were recovered in the Con A-bound fraction. The bound fraction was eluted with 0.25 M α -D-mannopyranoside. Immunoprecipitation was then carried out, as described supra, and the precipitates were separated on NaDodSO₄/PAGE, also as discussed supra.

Those cells transfected only with pFAP.38 produced FAP α , but not FAP β (determined from mAb F19 immunoprecipitates). They also produce no CD26 antigen (tested with EF-1). Those cells transfected with pCD26 alone produce CD26 but no FAP α . Cotransfectants produce CD26 and FAP α /FAP β heteromers, as determined in the mAb F19 precipitates. This result provides direct evidence that FAP β is a CD26 gene product.

Example 8

It has been observed previously that some cultured human cell types coexpress FAP α and CD26, and show FAP α /CD26 heteromer formation. *In vivo* distribution patterns of FAP α and CD26, however, as determined in previous immunohistochemical studies, appeared to be non-overlapping. (See Rettig et al., Proc. Natl. Acad. Sci. USA 85: 3110-3114 (1988); Garin-Chesa et al., Proc. Natl. Acad. Sci. USA 87: 7235-7329 (1990); Rettig et al., Canc. Res. 53: 3327-3335 (1993); Stein et al., in Knapp et al., eds. Leukocyte typing IV-white cell differentiation antigens, pp 412-415 (Oxford University Press, N.Y. 1989), pp. 412-415; Möbius et al., J. Exp. Immunol. 74: 431-437 (1988)). In view of the potential significance of FAP α /CD26 coassociation, tissue distribution was reexamined, via side by side immunohistochemical staining of normal tissues and lesional tissues known to contain FAP α ⁺ fibroblasts or FAP α ⁺ malignant cells.

To test the samples, they were embedded in OCT compound, frozen in isopentane precooled in liquid nitrogen, and stored at -70°C until used. Five micrometer thick sections were cut, mounted on poly-L-lysine coated slides, air dried, and fixed in cold acetone (4°C, for 10 minutes). The sections were then tested with mAbs (10-20 ug/ml), using the well known avidin-biotin immuno-peroxidase method, as described by, e.g., Garin-Chesa et al., J. Histochem. Cytochem. 37: 1767-

1776 (1989); Garin-Chesa et al., Proc. Natl. Acad. Sci. USA 87: 7235-7239 (1990); Rettig et al., Canc. Res. 53: 3327-3335 (1993); Garin-Chesa et al., Am. J. Pathol. 142: 557-567.

The results are shown in figure 2. Breast, colorectal, pancreas and lung carcinomas showed strong expression of FAP α and no CD26 was found (see figures 2A and 2B). Five FAP α sarcomas, including malignant fibrous histiocytoma (figures 2C and 2D), were tested, and there was no expression of CD26. Examination of reactive fibroblasts of healing dermal wounds (figures 2E, 2F), showed abundant expression of both FAP α and CD26. The three renal carcinomas tested (figures 2G, 2H), showed expression of CD26 in malignant epithelium. FAP α was absent from malignant epithelial cells, and showed low expression in the stroma of these carcinomas.

Example 9

A mammalian cell line, transfected with a FAP α encoding cDNA, was prepared.

Human embryonic kidney cell line 293 is well known and widely available from, e.g., the American Type Culture Collection.

Samples of 293 were maintained, in an incubator, at 37°C, in an atmosphere of 95% air, and 5% CO₂. The cells were cultured in a 50:50 mixture of Dulbecco's modified minimal essential medium and Ham's F12 medium, augmented with 10% fetal bovine serum, penicillin and streptomycin. Following the procedures described by Ustar et al., Eur. Mol. Biol. J. 1991, and/or Park et al., J. Biol. Chem. 169: 25646-25654 (1994), both of which are incorporated by reference, cDNA for FAP α (i.e., SEQ ID NO: 1), was transfected into the 293 cells. Details of the cDNA vector are provided, supra (pFAP.38). Transfectants were selected for resistance to antibiotics (200 ug/ml Geneticin), and were then maintained in selection medium, containing Geneticin.

Individual colonies of resistant cells were picked, grown to confluence in 6 well tissue culture plates, and were tested for FAP α expression in an immunofluorescence assay (IFA), using FAP α specific monoclonal antibody F19 as described

supra.

Those colonies which expressed FAP α were expanded, and monitored by indirect IFA and cytofluorometric analysis, also as set forth, supra.

The IFAs were positive for the transfectants, referred to hereafter as cell line 293-FAP, but were negative for parental line 293.

Example 10

In order to confirm that recombinant FAP α was, in fact, being produced, a series of immunoprecipitation experiments were carried out. These followed the methods of Park, et al., supra, and Rettig et al., Canc. Res. 53: 3327-3335 (1993), both of which are incorporated by reference. Essentially, ³⁵[S] methionine labelled cell extracts were combined with monoclonal antibody F19, in the manner described supra. Precipitates were then boiled in extraction buffer and run on SDS-PAGE gels, using, as a negative control, mouse IgG1. Both cell line 293-FAP, and non transfected line 293 were tested. The results indicated clearly, that recombinant FAP α was produced by the transfected cell line 293-FAP. This was determined by immunoprecipitation analyses, using FAP α specific monoclonal antibody F19.

Example 11

The ability to produce recombinant FAP α permitted further study of the molecule's properties. Specifically, given the structural features outlined in the prior examples, experiments were designed to determine if FAP α possesses enzymatic activities. The experiments were designed to test whether or not FAP α had extracellular matrix (ECM) protein degrading activity.

Extracts of 293-FAP cells were prepared, using an extraction buffer (0.15M NaCl, 0.05M Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 percent Triton X-114), were cleared by centrifugation (4,000xg, 10 minutes at 4°C), and phase partitioned at 37°C for 10-20 minutes. This was followed by further centrifugation (4000xg, 20 minutes at 20-25°C). Detergent phases were diluted with buffer (0.15 M NaCl, 0.05 M Tris-HCl

pH 7.4, 5 mM CaCl_2 , 5 mM MgCl_2 , 0.75% Empigen BB), and separated on concanavalin A-Sepharose following Rettig et al., *supra*. Any concanavalin A bound fractions were eluted with 0.25M methyl- α -D-mannopyranoside in elution buffer 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, 5mM CaCl_2 , 5 mM MgCl_2 , 0.1% Triton X-100), mixed with zymography sample buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.01% bromophenol blue), at a 3:1 ratio, and used for further analysis.

Aliquots of sample were loaded onto polyacrylamide gels containing 0.1% of either of gelatin or casein. Electrophoresis was then carried out in a Biorad Mini-Protein II system, at 20 mA constant current for 1.5 - 2 hours, until the bromophenol blue dye fronts of samples had reached the lower end of the gel. The gel was removed and incubated for one hour at 20-25°C in a 2.5% aqueous solution of Triton X-100 on a rotary shaker. The Triton X-100 solution was decanted, and replaced with enzyme buffer (0.05M Tris-HCl, pH 7.5, 0.2M NaCl, 5 mM CaCl_2 , 5 mM MgCl_2 , 0.02% Brij 35). The gel was then incubated at 37°C or 41°C, followed by staining or destaining at room temperature. Gels were stained with 0.5% of Coomassie Brilliant Blue G-250 in an aqueous solution of 30% methanol and 10% acetic acid for 15, 30, and 60 minutes, respectively. Subsequently, gels were incubated for 15 minutes in an aqueous solution of 30% CH_3OH and 5% glycerol, followed by drying between sheets of cellophane.

Gelatinase activity was evaluated in accordance with Kleiner et al., Anal. Biochem. 218: 325-329 (1994), incorporated by reference in its entirety. This is a routine assay used to determine whether or not a protease capable of digesting gelatin is present. Labelled molecular weight standard were run on the same gels, under reducing conditions, for molecular weight determinations.

Proteolytic activity for defined amino acid sequence motifs were tested, using a well known membrane overlay assay. See Smith et al, Histochem. J. 24(9): 637-647 (1992), incorporated by reference. Substrates were Ala-Pro-7-amino-4-trifluoromethyl coumarin, Gly-Pro-7-amino-4-trifluoromethyl

coumarin, and Lys-Pro-7-amino-4-trifluoromethyl coumarin.

The results of these experiments are depicted, in part, in figure 3. This figure shows zymographic detection of gelatin degrading activity, in the cell extracts. See Kleiner et al., supra. A protein species of approximately 170 kilodaltons, as determined by SDS-PAGE, was observed to have gelatin degrading activity. This species, which was found in the 293-FAP cell line, but not in untransfected 293 cells, is thus identified as FAP α . The molecular weight is consistent with a dimer, i.e., a dimeric FAP α molecule.

The proteolytic activity described herein where gelatin is the substrate, was not observed when casein was the substrate.

Example 12

Further studies were then undertaken in order to characterize the 170 kD FAP α dimer further. Specifically, the experiments described in example 11 were repeated, except that 5% of 2-mercaptoethanol or 5 mM iodoacetamide was added to the extracts prior to SDS-PAGE, or ethylenediamine N,N,N',N'-tetraacetic acid (10 mM) was added to the incubation buffer used for gelatin zymography. None of these treatments abolished the enzymatic activity. In contrast, heating at 100°C for five minutes prior to SDS-polyacrylamide gel electrophoresis abolished the gelatin-degrading activity.

Further work, using a membrane overlay assay, described by, e.g., Smith et al., Histochem J. 24(9): 643-647 (1992), incorporated by reference, revealed that the FAP α dimers were able to cleave all of the Ala-Pro, Gly-Pro, and Lys-Pro dipeptides tested.

In further experiments, a fusion protein was produced which comprised the extracellular domains of both FAP α and murine CD8 proteins. This chimeric protein was produced in a baculovirus system in insect cells. The chimeric protein exhibited the same enzymatic activity as FAP α , using the model discussed supra.

Example 13

Two quantitative assays for FAP α enzyme activity were developed using Ala-Pro-7-amino-4-trifluoromethyl coumarin (Ala-Pro-AFC) as the substrate. In the first assay format, membrane extracts of FAP α -expressing cells were mixed with a 5-10 fold volume of reaction buffer (100mM NaCl, 100mM Tris pH 7.8), and added to an equal volume of 0.5mM Ala-Pro-AFC in reaction buffer followed by an incubation for one hour at 37°C. Release of free AFC was then measured in a fluorimeter using a 395nm excitation / 530nm emission filter set. The membrane extracts analyzed in this assay format were derived from either 293-FAP α cells (293 cells stably transfected with vector FAP.38 described supra) or HT1080-FAP α cells (HT1080 cells stably transfected with vector FAP.38). Negative control experiments assessing FAP α -specific activities were carried out with membrane extracts prepared from the respective parental 293 or HT1080 cell lines. In the second assay, FAP α was isolated from 293-FAP α or HT1080-FAP α membrane extracts via an antibody specific for FAP α . Ninety-six well ELISA plates were coated overnight at 4°C with 1 μ g/ml F19 monoclonal antibody in phosphate-buffered saline (PBS). In the case of CD8-FAP α discussed infra plates were coated with F19 antibody as above or with 1 μ g/ml rat anti-mouse CD8 overnight at 4°C. Wells were then washed with wash buffer (PBS, 0.1% Tween 20). Excess binding sites were blocked with blocking buffer (5% bovine serum albumin in PBS) for 1 hour at room temperature. Blocking buffer was removed; membrane extracts of 293-FAP α expressing cells or control cells were added and incubated for 1 hour at room temperature. The unbound material was removed, wells were washed with wash buffer, and FAP α activity was assayed using 100 μ l Ala-Pro-AFC (0.5 mM Ala-Pro-AFC in reaction buffer) for one hour at 37°C. Release of free AFC was measured as above. Binding of mab F19 to FAP α did not measurably affect its enzymatic activity.

Example 14

Using assays for FAP α enzyme activity, described supra an inhibitor of FAP α enzymatic activity has been identified. This inhibitor is (S)-Valylpyrrolidine-2(R)-boronic acid (Snow

et al., J. Am. Chem Soc.(1994) 116:10860-10869), referred to here as ValboroPro. ValboroPro inhibits cleavage of Ala-Pro-AFC by FAP α with an IC₅₀ of 0.11 μ M. ValboroPro also inhibits the gelatinolytic activity of FAP α at a concentration of 100 μ M. The specificity of ValboroPro for FAP α was demonstrated in tests with an unrelated serine protease, trypsin. No inhibition of bovine trypsin by ValboroPro (up to 100 μ M) was observed when assayed with carbobenzoxy-L-valinyl-glycinyl-L-arginyl-4-nitranilide acetate as substrate.

Example 15

The identification of specific, structural requirements for the enzymatic activities of FAP α facilitates the development of molecules which can bind to and/or inhibit FAP α . To examine whether the serine residue at position 624 of the predicted amino acid sequence of FAP α polypeptide is critical for its enzymatic function, site-directed mutagenesis according to Zoller, et al DNA 3:479-488 (1984) was performed using standard polymerase chain reaction methods. The TCC codon coding for serine 624 in the FAP α cDNA was replaced with GCG, resulting in alanine at this position. The altered DNA was reintroduced into the FAP.38 vector and transfected into 293 cells as described *supra*. Geneticin-resistant colonies were selected and examined by indirect IFA for FAP α expression using mAb F19 as well as other FAP α specific antibodies described by Rettig, et al., J. Cancer 58:385-392 (1994) as set forth, *supra*. No differences in binding of the anti-FAP α antibodies to the mutant FAP α expressing cells were observed as compared to wild type FAP α transfected cells. The presence of the mutation was confirmed through amplification of genomic DNA and restriction enzyme digestion performed with several clones of transfected cells. To assess the enzymatic activity of mutant FAP α , the following tests were performed. Membrane extracts were prepared from three independent positive clones and equal amounts of FAP α protein (as determined in a double-determinate ELISA assay using two anti-FAP α antibodies that recognize distinct FAP α epitopes) were examined in the gelatinolytic and Ala-Pro-AFC capture assays.

Both the gelatinolytic activity and the activity in the capture assay of isolated mutant FAP α were reduced to undetectable levels compared to wild type FAP α , confirming the role of the canonical serine in the catalytic triad for both observed enzymatic activities.

Example 16

A fusion protein was generated to obtain secreted, water-soluble FAP α enzyme. In this fusion protein, the extracellular domain of CD8, consisting of the first 189 amino acids of murine CD8, was linked to the extracellular domain of FAP α (amino acids 27 to 760), as described by Lane et al., J. Exp. Med. 177:1209 (1993) using standard polymerase chain reaction protocols and inserted in commercially available pVL1393 vector. Transfection of Sf9 cells with this vector and amplification of the resulting recombinant baculovirus were performed as described (Baculovirus Expression Vectors, O'Reilly, Miller, and Luckow, Oxford University Press, 1994). The CD8-FAP fusion protein was isolated in a two step purification from the spent medium of High Five™ cells infected with CD8-FAP α baculovirus for four days. Cells and virus were removed by ultracentrifugation, the supernatant was passed through a column containing Heparin-Sepharose (Pharmacia) and eluted stepwise with 0.6, 1.0, and 2.0 M NaCl in 10mM phosphate, pH 7. Active fractions from the 1.0 and 2.0 M eluates were pooled and concentrated using an YM-10 filter and 26/60 Superdex-200 gel filtration column. Activity was observed in a high molecular weight peak which, when subjected to N-terminal gas phase sequencing, was confirmed to be CD8-FAP α . In gelatinolytic assays, activity greater than 200kD in the gelatinolytic assay was detected when purified CD8-FAP α was tested, consistent with the higher predicted molecular weight of the fusion protein.

Example 17

The presence of structural and functional homologues in non-human species has been ascertained. For example, the cDNA for mouse FAP α has been cloned and characterized. Examination of the predicted amino acid sequence of the homologous mouse

FAP α cDNA sequence (EMBL accession number Y10007) reveals a high degree of conservation of FAP α across species. The two proteins are 89% identical and the catalytic triad is conserved between human FAP α and mouse FAP α . The high degree of conservation and similar tissue expression suggests that FAP α from nonhuman sources may be functionally equivalent to human FAP α . This conclusion is confirmed by the finding that a CDS-murine FAP α fusion protein similar in design to CDS-human FAP α also demonstrates the expected dipeptidylpeptidase enzymatic activity using Ala-Pro-AFC as substrate.

The foregoing examples describe an isolated nucleic acid molecule which codes for fibroblast activating protein alpha ("FAP α "), as well as dimeric forms of the molecule, and uses thereof. The expression product of the sequence in COS-1 is a protein which, on SDS-PAGE of boiled samples, shows a molecular weight of about 88 kd. Deduced amino acid sequence, as provided in SEQ ID NO: 1, for one form of the molecule, yields a molecular weight of about 88 kd.

It should be noted that there is an apparent discrepancy in molecular weight in that the COS-1 isolate is glycosylated, while molecular weight from deduced amino acid sequences does not account for glycosylation. Membrane proteins are known to exhibit aberrant migration in gel systems, however, which may explain the difference observed here.

Also a part of the invention are chimeric and fusion proteins, which comprise a portion of FAP α which contain the molecule's catalytic domain, and additional, non FAP α components. The FAP α catalytic domain per se is also a part of the invention.

It is to be understood that, as described, FAP α may be glycosylated, with the type and amount of glycosylation varying, depending upon the type of cell expressing the molecule. The experiment described herein shows this. This is also true for the dimeric form of the molecule, first described herein, having a molecular weight of about 170 kilodaltons as determined by SDS-PAGE of unboiled samples.

The invention also comprehends the production of

expression vectors useful in producing the FAP α molecule. In their broadest aspect, these vectors comprise the entire FAP α coding sequence or portions thereof, operably linked to a promoter. Additional elements may be a part of the expression vector, such as protein domains fused to the FAP α protein or protein portions ("fusion protein") genes which confer antibiotic resistance, amplifiable genes, and so forth.

The coding sequences and vectors may also be used to prepare cell lines, wherein the coding sequence or expression vector is used to transfect or to transform a recipient host. The type of cell used may be prokaryotic, such as *E. coli*, or eukaryotes, such as yeast, CHO, COS, or other cell types.

The identification of nucleic acid molecules such as that set forth in SEQ ID NO: 1 also enables the artisan to identify and to isolate those nucleic acid molecules which hybridize to it under stringent conditions. "Stringent condition" as used herein, refers to those parameters set forth supra, whereby both murine and hamster sequences were also identified. It will be recognized by the skilled artisan that these conditions afford a degree of stringency which can be achieved using parameters which vary from those recited. Such variance is apprehended by the expression "stringent conditions".

The ability of nucleic acid molecules to hybridize to complementary molecules also enables the artisan to identify cells which express FAP α , via the use of a nucleic acid hybridization assay. One may use the sequences described in the invention to hybridize to complementary sequences, and thus identify them. In this way, one can target mRNA, e.g., which is present in any cell expressing the FAP α molecule.

It is of course understood that the nucleic acid molecules of the invention are also useful in the production of recombinant FAP α , in both monomeric and dimeric form. The examples clearly show that host cells are capable of assembling the dimeric forms. The recombinant protein may be used, e.g., as a source of an immunogen for generation of antibodies akin to known mAb F19, and with the same uses. Similarly, the recombinant protein, and/or cells which express

the molecule on their surface, may be used in assays to determine antagonists, agonists, or other molecules which interact with molecules having FAP α activity. Such substances may be, but are not necessarily limited to, substrates, inhibiting molecules, antibodies, and so forth. The molecules having FAP α activity may be, e.g., the monomeric or dimeric forms of FAP α , derivatives containing the catalytic domain, and so forth. The molecule having FAP α activity may be pure, or in the form of a cell extract, such as a transformed or transfected cell, which has received an FAP α active gene. Both prokaryotes and eukaryotes may be used. This last feature of the invention should be considered in light of the observed structural resemblances to membrane bound enzymes. This type of molecule is associated with certain properties which need not be described in detail here. It will suffice to say that inhibition or potentiation of these properties as associated with FAP α is a feature of this invention. For example, one may identify substrates or the substrate for FAP α molecules, via the use of recombinant cells or recombinant FAP α per se. The substrates can be modified to improve their effect, to lessen their effect, or simply to label them with detectable signals so that they can be used, e.g., to identify cells which express FAP α . Study of the interaction of substrate and FAP α , as well as that between FAP α and any molecule whatsoever, can be used to develop and/or to identify agonists and antagonists of the FAP α molecule.

Also a feature of the invention are isolated, dimeric FAP α

molecules which have a molecular weight of about 170 kilodaltons as determined by SDS-PAGE, their use as an enzymatic cleaving agent, and other uses as are described herein. Enzymatically active forms of FAP α may also be produced as recombinant fusion proteins, such as soluble fusion proteins comprising the catalytic domain of FAP α and other protein domains with suitable biochemical properties, including secretory signals, protease cleavage sites, tags for purification, and other elements known to the artisan.

Exemplary are CD8 peptide sequences, such as are described supra. The fact that FAP α has particular properties, as described herein, permits the identification of the molecule on cells expressing them. In turn, because the FAP α molecule is associated with tumors and tumor stromal cells, targeting of FAP α with therapeutic agents serves as a way to treat cancerous or precancerous condition, by administering sufficient therapeutic agent to alleviate cancer load.

The experiments showing the proteolytic properties of FAP α lead to yet a further aspect of the invention. It is well known that proteases which degrade extracellular matrix, or "ECM" proteins have an important role on certain aspects of tumor growth, including their effect on tumor cell invasion, tumor blood vessel formation (i.e., neoangiogenesis), and tumor metastasis. Collagens are of special interest vis-a-vis the substrates of proteases, as the collagens are an important part of the ECM. The fact that FAP α digests ECM suggests a therapeutic role for inhibitors of the molecule. "Inhibitors", as used herein, refers to molecules which interfere with FAP α enzyme function. Specifically excluded from such inhibitors is the monoclonal antibody F19. This mAb is known to bind to but not inhibit the enzyme function of FAP α , and hence it is not an inhibitor. The art is quite well versed with respect to monoclonal antibodies which both bind to and inhibit enzymes. Further examples of such inhibitors would include, e.g., substrate derivatives, such as modified collagen molecules, which interfere with the active site or sites of the FAP α molecule. Other suitable inhibitors will be apparent to the skilled artisan, and need not be listed here. In addition, the recombinant FAP α proteins and FAP α -transfected cell lines described supra can be employed in an enzymatic screening assay, using the substrate described supra or other suitable substrates, to identify inhibitors from any compound library. The identification of substances which interact with FAP α active molecules thereby leads to therapeutic treatment of conditions where a subject exhibit abnormal FAP α activity. Specifically, an amount of an

appropriate substance, be it an inhibitor (e.g, a collagen derivative, S-Valyl-pyrrolidine-2(R)-boronic acid), an agonist or an antagonist is administered to a subject in an amount sufficient to normalize FAPa activity.

Other aspects of the invention will be clear to the skilled artisan, and need not be set forth here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

(1) GENERAL INFORMATION:

- (i) APPLICANTS: Zimmermann, Rainer; Park, John E.;
Rettig, Wolfgang; Old, Lloyd J.
- (ii) TITLE OF INVENTION: ISOLATED DIMERIC FIBROBLAST ACTIVATION
PROTEIN ALPHA, AND USES THEREOF
- (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Felte & Lynch
 - (B) STREET: 805 Third Avenue
 - (C) CITY: New York City
 - (D) STATE: New York
 - (E) COUNTRY: USA
 - (F) ZIP: 10022
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Diskette, 3.5 inch, 2.0 MB storage
 - (B) COMPUTER: IBM PS/2
 - (C) OPERATING SYSTEM: PC-DOS
 - (D) SOFTWARE: Wordperfect
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
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- (A) APPLICATION NUMBER: 08/619,280
 - (B) FILING DATE: 18-MARCH-1996
 - (C) CLASSIFICATION: 435
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- (A) APPLICATION NUMBER: 08/230,491
 - (B) FILING DATE: 20-APRIL-1994
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Hanson, Norman D.
 - (B) REGISTRATION NUMBER: 30,946
 - (C) REFERENCE/DOCKET NUMBER: LUD 5330.1-PCT
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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2815 Base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO: 1:

AAGAACGCCC CAAAATCTG TTTCTAATTT TACAGAAATC TTTTGAAACT TGGCAGGTA 60
TTCAAAAGTC CGTGGAAGA AAAAAACCTT GTCCTGGCTT CAGCTTCAA CTACAAAGAC 120
AGACTTGGTC CTTTCAACG GTTTTCACAG ATCCAGTGAC CCACGCTCTG AAGACAGAAT 180
TAGCTAATTT TCAAAAACAT CTGGAAGAAAT GAAGACTTGG GTAAAAATCG TATTTGGAGT 240
TGCCACCTCT GCTGTGCTTG CCTTATTGGT GATGTGCATT GTCTTACGCC CTTCAAGAGT 300
TCATAACTCT GAAGAAAATA CAATGAGAGC ACTCAGCTG AAGGATATTT TAAATGGAAC 360
ATTTTCTTAT AAAACATTTT TTCCAAACTG GATTCAGGA CAAGAATATC TTCATCAATC 420
TGCAGATAAC AATATAGTAC TTTATAATAT TGAACAGGA CAATCATATA CCATTTTGAG 480
TAATAGAACC ATGAAAAGTG TGAATGCTTC AAATTACGGC TTATCACCTG ATCGGCAATT 540
TGTATATCTA GAAAGTGATT ATTCAAAGCT TTGGAGATAC TCTTACACAG CAACATATTA 600
CATCTATGAC CTTAGCAATG GAGAAATTTG AAGAGGAAAT GAGCTTCCTC GTCCAATTCA 660
GTATTTATGC TGGTCGCCTG TTGGGAGTAA ATTAGCATAT GTCTATCAA ACAATATCTA 720
TTTGAAACAA AGACCAGGAG ATCCACCTTT TCAAATAACA TTTAATGGAA GAGAAAATAA 780
AATAJTAAAT GGAATCCCAG ACTGGGTTTA TGAAGAGGAA ATGCTTCCTA CAAATATGTC 840
TCTCTGGTGG TCTCCTAATG GAAAAATTTT GGCATATGCG GAATTTAATG ATAAGGATAT 900
ACCAATTATT GCCTATTCCT ATTATGGCGA TGAACAATAT CCTAGAACA TAAATATTCC 960
ATACCCAAAG GCTGGAGCTA AGAATCCCGT GTTTCGGATA TTTATTATCG ATACCACTTA 1020
CCCTGCGTAT GTAGGTCCCC AGGAAGTGCC GTTCCAGCA ATGATAGCCT CAAGTGATTA 1080
TTATTTAGT TGGCTCACGT GGGTTACTGA TGAACGAGTA TGTTCGAGT GGCTAAAAAG 1140
AGTCCAGAAT GTTTCGGTCC TGTCTATATG TGACTTCAGG GAAGACTGGC AGACATGGA 1200
TTGTCCAAAG ACCCAGGAGC ATATAGAAGA AAGCAGAACT GGATGGGCTG GTGGATTCTT 1260
TGTTTCAAGA CCAGTTTTC ACTATGATGC CATTTCGTAC TACAAAATAT TTAGTGACAA 1320
GGATGGCTAC AACATATTC ACTATATCAA AGACACTGTG GAAAATGCTA TTCAAATTAC 1380
AAGTGGCAAG TGGGAGGCCA TAAATATATT CAGAGTAACA CAGGATTCAC GTTTTATTTC 1440
TAGCAATGAA TTTGAAGAAT ACCCTGGAAG AAGAAACATC TACAGAATTA GCATTGGAAG 1500
CTATCCTCCA AGCAAGAAGT GTGTTACTTG CCATCTAAGG AAAGAAAGGT GCCAATATTA 1560
CACAGCAAGT TTCAGCGACT ACGCCAAGTA CTATGCACTT GTCTGCTACG GCCCAGGCAT 1620
CCCCATTTC ACCCTTCATG ATGGACGCAC TGATCAAGAA ATTAATATCC TGAAGAAAA 1680

25

CAAGGAATTG GAAATGCTT TGAAAAATAT CCAGCTGCCT AAAGAGGAAA TTAAGAAACT 1740
 TGAAGTAGAT GAAATTACTT TATGGTACAA GATGATTCTT CCTCCTCAAT TTGACAGATC 1800
 AAAGAAGTAT CCCTTGCTAA TTCAAGTGTA TGGTGGTCCC TGCAGTCAGA GTGTAAGGTC 1860
 TGTATTTGCT GTTAATTGGA TATCTTATCT TGCAAGTAAG GAAGGGATGG TCATTGCCTT 1920
 GGTGGATGGT CGAGGAACAG CTTTCCAAGG TGACAAACTC CTCTATGCAG TGTATCGAAA 1980
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 TTTCATTGAT GAAAAAAGAA TAGCCATATG GGGCTGGTCC TATGGAGGAT ACGTTTCATC 2100
 ACTGGCCCTT GCATCTGGAA CTGGTCTTTT CAAATGTGGT ATAGCAGTGG CTCCAAGTCTC 2160
 CAGCTGGGAA TATTACGCGT CTGTCTACAC AGAGAGATTG ATGGGTCTCC CAACAAAGGA 2220
 TGATAATCTT GAGCACTATA AGAATTCAAC TGTGATGGCA AGAGCAGAAT ATTCAGAAA 2280
 TGTAGACTAT CTTCTCATCC ACGGAACAGC AGATGATAAT GTGCACTTTC AAAACTCAGC 2340
 ACAGATTGCT AAAGCTCTGG TTAATGCACA AGTGGATTTC CAGGCAATGT GGTACTCTGA 2400
 CCAGAACCAC GGCTTATCCG GCCTGTCCAC GAACCACTTA TACACCCACA TGACCCACTT 2460
 CCTAAAGCAG TGTCTCTCTT TGTGAGACTA AAAACGATGC AGATGCAAGC CTGTATCAGA 2520
 ATCTGAAAAC CTTATATAAA CCCCTCAGAC AGTTTGCTTA TTTTATTTTT TATGTTGTAA 2580
 AATGCTAGTA TAAACAAACA AATTAATGTT GTTCTAAAGG CTGTTAAAAA AAAGATGAGG 2640
 ACTCAGAAGT TCAAGCTAAA TATTGTTTAC ATTTTCTGGT ACTCTGTGAA AGAAGAGAAA 2700
 AGGGAGTCAT GCATTTTGCT TTGGACACAG TGTTTTATCA CCTGTTTATT TGAAGAAAAA 2760
 TAATAAAGTC AGAAGTTCAA AAAAAAAAAA AAAAAAAAAA AAAGCGGCCG CTCGA 2815

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 760 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Lys Thr Trp Val Lys Ile Val Phe Gly Val Ala Thr Ser Ala Val
 5 10 15
 Leu Ala Leu Leu Val Met Cys Ile Val Leu Arg Pro Ser Arg Val His
 20 25 30
 Asn Ser Glu Glu Asn Thr Met Arg Ala Leu Thr Leu Lys Asp Ile Leu
 35 40 45
 Asn Gly Thr Phe Ser Tyr Lys Thr Phe Phe Pro Asn Trp Ile Ser Gly

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	50		55		60
	Gln Glu Tyr Leu His Gln Ser Ala Asp Asn Asn Ile Val Leu Tyr Asn				
	65		70		75
	Ile Glu Thr Gly Gln Ser Tyr Thr Ile Leu Ser Asn Arg Thr Met Lys				80
5		85		90	95
	Ser Val Asn Ala Ser Asn Tyr Gly Leu Ser Pro Asp Arg Gln Phe Val				
	100		105		110
	Tyr Leu Glu Ser Asp Tyr Ser Lys Leu Trp Arg Tyr Ser Tyr Thr Ala				
	115		120		125
10	Thr Tyr Tyr Ile Tyr Asp Leu Ser Asn Gly Glu Phe Val Arg Gly Asn				
	130		135		140
	Glu Leu Pro Arg Pro Ile Gln Tyr Leu Cys Trp Ser Pro Val Gly Ser				
	145		150		155
	Lys Leu Ala Tyr Val Tyr Gln Asn Asn Ile Tyr Leu Lys Gln Arg Pro				160
15		165		170	175
	Gly Asp Pro Pro Phe Gln Ile Thr Phe Asn Gly Arg Glu Asn Lys Ile				
	180		185		190
	Phe Asn Gly Ile Pro Asp Trp Val Tyr Glu Glu Glu Met Leu Pro Thr				
	195		200		205
20	Lys Tyr Ala Leu Trp Trp Ser Pro Asn Gly Lys Phe Leu Ala Tyr Ala				
	210		215		220
	Glu Phe Asn Asp Lys Asp Ile Pro Val Ile Ala Tyr Ser Tyr Tyr Gly				
	225		230		235
	Asp Glu Gln Tyr Pro Arg Thr Ile Asn Ile Pro Tyr Pro Lys Ala Gly				240
		245		250	255
25	Ala Lys Asn Pro Val Val Arg Ile Phe Ile Ile Asp Thr Thr Tyr Pro				
	260		265		270
	Ala Tyr Val Gly Pro Gln Glu Val Pro Val Pro Ala Met Ile Ala Ser				
	275		280		285
30	Ser Asp Tyr Tyr Phe Ser Trp Leu Thr Trp Val Thr Asp Glu Arg Val				
	290		295		300
	Cys Leu Gln Trp Leu Lys Arg Val Gln Asn Val Ser Val Leu Ser Ile				
	305		310		315
	Cys Asp Phe Arg Glu Asp Trp Gln Thr Trp Asp Cys Pro Lys Thr Gln				320
		325		330	335
35	Glu His Ile Glu Glu Ser Arg Thr Gly Trp Ala Gly Gly Phe Phe Val				
	340		345		350

Ser Arg Pro Val Phe Ser Tyr Asp Ala Ile Ser Tyr Tyr Lys Ile Phe
 355 360 365
 Ser Asp Lys Asp Gly Tyr Lys His Ile His Tyr Ile Lys Asp Thr Val
 370 375 380
 Glu Asn Ala Ile Gln Ile Thr Ser Gly Lys Trp Glu Ala Ile Asn Ile
 385 390 395 400
 Phe Arg Val Thr Gln Asp Ser Leu Phe Tyr Ser Ser Asn Glu Phe Glu
 405 410 415
 Glu Tyr Pro Gly Arg Arg Asn Ile Tyr Arg Ile Ser Ile Gly Ser Tyr
 420 425 430
 Pro Pro Ser Lys Lys Cys Val Thr Cys His Leu Arg Lys Glu Arg Cys
 435 440 445
 Gln Tyr Tyr Thr Ala Ser Phe Ser Asp Tyr Ala Lys Tyr Tyr Ala Leu
 450 455 460
 Val Cys Tyr Gly Pro Gly Ile Pro Ile Ser Thr Leu His Asp Gly Arg
 465 470 475 480
 Thr Asp Gln Glu Ile Lys Ile Leu Glu Glu Asn Lys Glu Leu Glu Asn
 485 490 495
 Ala Leu Lys Asn Ile Gln Leu Pro Lys Glu Glu Ile Lys Lys Leu Glu
 500 505 510
 Val Asp Glu Ile Thr Leu Trp Tyr Lys Met Ile Leu Pro Pro Gln Phe
 515 520 525
 Asp Arg Ser Lys Lys Tyr Pro Leu Leu Ile Gln Val Tyr Gly Gly Pro
 530 535 540
 Cys Ser Gln Ser Val Arg Ser Val Phe Ala Val Asn Trp Ile Ser Tyr
 545 550 555 560
 Leu Ala Ser Lys Glu Gly Met Val Ile Ala Leu Val Asp Gly Arg Gly
 565 570 575
 Thr Ala Phe Gln Gly Asp Lys Leu Leu Tyr Ala Val Tyr Arg Lys Leu
 580 585 590
 Gly Val Tyr Glu Val Glu Asp Gln Ile Thr Ala Val Arg Lys Phe Ile
 595 600 605
 Glu Met Gly Phe Ile Asp Glu Lys Arg Ile Ala Ile Trp Gly Trp Ser
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 625 630 635 640
 Phe Lys Cys Gly Ile Ala Val Ala Pro Val Ser Ser Trp Glu Tyr Tyr

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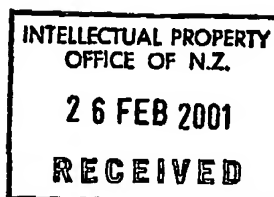
645										650					655									
Ala	Ser	Val	Tyr	Thr	Glu	Arg	Phe	Met	Gly	Leu	Pro	Thr	Lys	Asp	Asp									
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Asn	Leu	Glu	His	Tyr	Lys	Asn	Ser	Thr	Val	Met	Ala	Arg	Ala	Glu	Tyr									
675										680					685									
Phe	Arg	Asn	Val	Asp	Tyr	Leu	Leu	Ile	His	Gly	Thr	Ala	Asp	Asp	Asn									
690										695					700									
Val	His	Phe	Gln	Asn	Ser	Ala	Gln	Ile	Ala	Lys	Ala	Leu	Val	Asn	Ala									
705										710					715					720				
Gln	Val	Asp	Phe	Gln	Ala	Met	Trp	Tyr	Ser	Asp	Gln	Asn	His	Gly	Leu									
725										730					735									
Ser	Gly	Leu	Ser	Thr	Asn	His	Leu	Tyr	Thr	His	Met	Thr	His	Phe	Leu									
740										745					750									
Lys	Gln	Cys	Phe	Ser	Leu	Ser	Asp																	
755										760														

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WE CLAIM:

1. Use of an inhibitor of FAP α activity which interacts with molecules having FAP α activity, said inhibitor being a collagen derivative, in the manufacture of a medicament for treating a subject with a pathological condition characterised by abnormal FAP α activity.
2. Use according to claim 1, wherein said inhibitor is (S) - Valyl-pyrrolidine-2(R) - boronic acid.
3. Use according to claim 1 and substantially as herein described with reference to any one of the examples.



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FIG. 1

FAP	1	MKTWVKIVFGV*ATSAVLALLVMCIVLRPSRVHNSEENTMRALTLKDILN	49
CD26	1	---PW-VLL-LLGAA-LVTIITVPV--LNKGTDDATADSRKTY--T-Y-K	50
FAP	50	GTFSYKTFPPNWIISGOEYLHQSDANNIVLYNIETGQSYTILSNRTMKSV*	98
CD26	51	N-YRL-LYSLR---DH---YKQ*E---LVF-A-Y-N-SVF-E-S-FDEFG	99
FAP	99	*NASNYGLSPDRQFVYLESDYSKLVRYSYTATYYIYDLSNGEFVRGNELP	147
CD26	100	HSIND-SI---G---IL--YN-V-Q--H-----S-D-----NKRQLITEERI-	149
		<u>fap-1</u>	
FAP	148	RPIQYLCWSPVGSKLAYVYQNNIYLKQRPDPPFQITFNGRENKIFNGIP	197
CD26	150	NNT-WVT-----E-----WN-D--V-IE-NL-SYR--WT-K-DI-Y---T	199
		<u>fap-2</u>	
FAP	198	DWVYEEEMLPKTYALWWSPNGKFLAYAEFNDKRDIPVIAYSYYGDE**QYP	245
CD26	200	-----VFSAYS-----T-----Q---TEV-L-E--F-S--SL---	249
FAP	246	RTINIPYPKAGAKNPVVRIFIIDT***TYPAYVGPQEVFPVPMIASSDY	292
CD26	250	K-VRV-----V--T-KF-VVN-DSLSSVTNATSIQITA--SMLIG-H-	299
FAP	293	FSWLTWVTDERVCLQWLKRVONVSVLSICDFREDWQWDCPKTQEHIEES	342
CD26	300	LCDV--A-Q--IS----R-I--Y--MD---YD-SSGR-N-LVARQ---M-	349
FAP	343	RTGWAGGFFVSRPVFSYDAISYYKIPSDKDGKHIHYIKDTVENAIQITS	392
CD26	350	T---V-R-RP-E-H-TL-GN-F---I-NEE--R--C-FQIDKKDCTF--K	399
FAP	393	GKWEAINIFRVTQDSLFSYSSNEFEYPPGRNRIYRISIGSYPPSKKCVTCH	442
CD26	400	-T--V-G-EAL-S-Y-Y-I---YKGM--G--L-K-QLSD-T*KVT-LS-E	448
FAP	443	LRKERCQYYTASFSDYAKYYALVCYGPPISTLHDGRTDQETKILEENK	492
CD26	449	-NP-----SV---KE---Q-R-S---L-LY---SSVN-KGLRV--D-S	498
		<u>fap-3</u>	
FAP	493	ELENALKNIQLPKKEIKLEVDEITLWYKMLPPOFDRSKKYPLLIQVYG	542
CD26	499	A-DKM-Q-V-M-SKKLDFIILN-TKF--Q-----H--K-----LD--A	548
FAP	543	GPCSQSVRSVFAVNWISYLASKEGMVIALVDGRGTAFQGDKLLYAVYRKL	592
CD26	549	-----KADT--RL--AT-----T-NIIV-SF-----SGY-----IMH-IN-R-	598
FAP	593	GVYEVEDQITAVRKFIEMGFIDEKRIAIWGSYEIRFITGPCIWNWSFQM	642
CD26	599	-TF-----E-A-Q-SK---V-N-----GGYVTSMVLGSGSVGFK	648
FAP	643	WYSSGSSLQLGILRVCLERE*IHGSPNKDDNLEHYKNSTVMARAEYFRNV	691
CD26	649	CGIAPVPSRWEYYSVYT-RYM-L-TPE---D--R-----S---N-KQ-	698
FAP	692	DYLLIHGTADDNVHFQNSAQIAKALVNAQVDFQAMWYSDQNHGLSGLSTN	741
CD26	699	E-----Q-----S-----DVG-----T-ED--IASSTAH	748
FAP	742	*HLYTHMTHFLKQCFSLSD	
CD26	749	Q-I-----S--I-----P	

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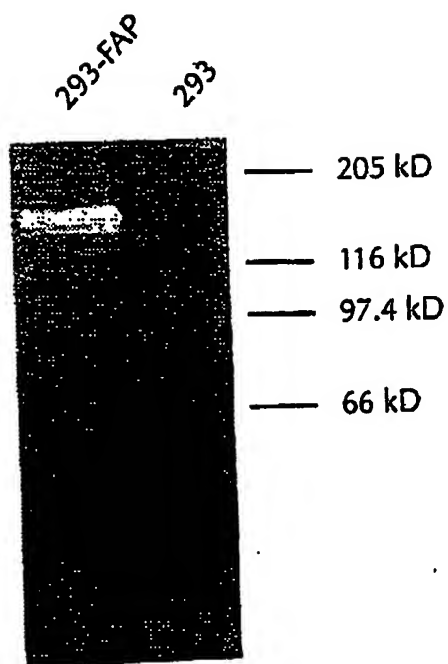
FIG. 2

	Breast Cancer	MFH	Healing Wound	Renal Cancer
FAP α	A \oplus	C \oplus	E \oplus	G \ominus
CD26	B \ominus	D \ominus	F \oplus	H \oplus

Immunohistochemistry (See Kodachromes)

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FIG. 3



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